Chemokine Receptors of T Cells and of B Cells in Lymphatic Filarial Infection: A Role for CCR9 in Pathogenesis

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We examined the expression of chemokine receptors on the surfaces of T cells and B cells from 27 individuals either with lymphatic filarial disease (lymphedema), with the asymptomatic or subclinical form of filarial infection, or without filarial infection. Individuals with lymphedema exhibited increased percentages of CCR9-expressing T cells and CCR9-expressing B cells and decreased percentages of both CXCR1-and-CXCR3–expressing T cells and CXCR1-and-CXCR3–expressing B cells, compared with asymptomatic or uninfected individuals. A significant correlation was found between the grade of lymphedema and the percentage of CCR9-expressing T cells and CCR9-expressing B cells. The percentages of CCR9-expressing T cells and CCR9-expressing B cells from patients with lymphedema was significantly up-regulated in response to live, infective-stage larvae of *Brugia malayi* but not to microfilariae of this parasite. Finally, individuals with lymphedema had significantly higher concentrations of interleukin-8, macrophage inflammatory protein (MIP)–1α, MIP-1β, monocyte chemotactic protein 1, thymus-and-activation–regulated chemokine, and interferon-inducible protein 10 in their serum than did uninfected individuals. These results suggest that chemokine receptors (particularly CCR9) are involved in the pathogenesis of lymphatic filarial disease and that trafficking of particular cellular subsets may influence clinical outcome.

The major pathological consequences of infection with the filarial parasite *Wuchereria bancrofti* are hydrocele, lymphedema, and, at the disease's most extreme stage, elephantiasis [1]. The development of filarial pathology is thought to be a multifaceted process in which extrinsic factors (high numbers of parasites in the body, transmission of a large number of larvae, and secondary bacterial infections) and intrinsic factors (host immune responses) play important roles [2, 3]. Data supporting the role for host T cells and host B cells in the pathogenesis of filarial disease come from both animal models and clinical studies of humans. Infection of either C3H/HeN nude mice [4] or BALB/c SCID mice [5]

with *Brugia malayi* parasites—which are phylogenetically similar to *W. bancrofti* parasites—resulted in lymphatic dilatation and lymphangitis but not in lymphatic obstruction. Only after these mice were given donor T cells and B cells did they develop obstructive pathology that included massive lymph thrombi and interstitial infiltrates [5, 6]. In human filarial disease (lymphedema), examination of inflammatory responses in local tissues reveals an abnormal T cell infiltrate, expression of the cell-surface markers HLA-DR and vascular cell adhesion molecule 1, and T cells with a bias for specific antigens [7, 8].

Recruitment of T cells and B cells to the site of infection, therefore, represents an important step in the pathogenesis of lymphedema. By promoting both chemotaxis and activation of integrins, chemokine receptors control these homing events of T cells and of B cells; thus, the expression of chemokine receptors defines distinct trophic and functional properties for T cells and for B cells [9]. Indeed, altered expression of

Table 1. Characteristics of study population.

	CP (n = 9)	CA+ (n = 9)	UN (n = 9)
Age, median (range), years	50 (40–58)	36 (27–55)	28 (25–50)
Men/women, no.	3/6	6/3	7/2
Pathology	Lymphedema grades 1–4[a]	None	None
Level of *Wuchereria bancrofti* circulating antigen, median, U/mL	<32–2149 (< 32)	254–49,069 (6429)	<32 (<32)

NOTE. The lower limit of detection in the assay was 32 U/mL. CP, filaria-infected individuals with chronic pathology/lymphedema; CA+, chronically infected but asymptomatic individuals; UN, uninfected individuals.

[a] The illness of each CP individual was classified according to the standard 4 grades that have been established by the World Health Organization.

chemokine receptors is associated with the development of disease in some parasitic infections, such as schistosomiasis [10] and Chagas disease [11]. In addition, chemokine receptors have been implicated in the pathogenesis of rheumatoid arthritis, multiple sclerosis, atopic dermatitis, Crohn disease, and allergic asthma [12].

Therefore, we examined the expression of chemokine receptors (both CCR and CXCR) on the surface of T cells and on B cells in filaria-infected individuals with chronic pathology/lymphedema (CP) and compared this expression to that seen in chronically infected but asymptomatic individuals (CA+) and to that seen in uninfected individuals (UN). Our data suggest that CCR9-expressing T cells and CCR9-expressing B cells play a role in the mediation of the lymphatic pathology associated with filarial infections.

SUBJECTS, MATERIALS, AND METHODS

Study population. We studied a cohort of 27 individuals in Tamil Nadu, in southern India, where lymphatic filariasis is endemic (table 1). This cohort comprised 9 CP individuals, 9 CA+ individuals, and 9 UN individuals. The status of active infection was determined by the ICT filarial antigen test (Bi-

nax), by the Trop Bio Og4C3 ELISA (TropBio), and by clinical evaluation of pathology. Each UN individual was negative for filarial antigens and had no evidence of lymphedema. Each CA+ individual was positive for filarial antigens and had no clinical evidence of lymphatic pathology. The illness of each CP individual was classified according to the standard 4 grades that have been established by the World Health Organization (WHO) [13]: grade 1, pitting edema that is reversible by elevation of the affected limb; grade 2, pitting/nonpitting edema that is not reversible by elevation of the affected limb; grade 3, nonpitting edema that is not reversible by elevation of the affected limb and that is accompanied by thickening of the skin; grade 4, nonpitting edema of the limb that is accompanied by fibrotic and verrucous skin changes (elephantiasis). Informed consent was obtained from patients who participated in the present study, and the human-experimentation guidelines of the US Department of Health and Human Services were followed in the conduct of clinical research.

Isolation of peripheral-blood lymphocytes. Samples of heparinized blood were collected, and peripheral-blood lymphocytes (PBLs) were isolated by Ficoll-diatrizoate gradient centrifugation (LSM; ICN Biomedicals). Erythrocytes were lysed

Table 2. Phenotypic analysis of T cells and B cells.

	CP (n = 9)	CA+ (n = 9)	UN (n = 9)
T cells (CD3+/CD56−)	70 (65–77)	68 (65–70)	71 (65–75)
CD69	1.6 (0.4–3.3)	1 (0.3–1.7)	1.3 (0.6–2.9)
CD25	16 (2.9–36.0)	16.6 (15.3–18.6)	14.6 (12.3–13.3)
CD71	1 (0.2–2.5)	2.2 (1.1–3.1)	1.5 (1.2–2.0)
CD45RA	36 (30–48)	39 (32–45)	36 (28–59)
CD45RO	41 (32–50)	39 (33–41)	43 (35–53)
B cells (CD19+)	10 (5–13)	18 (15–23)	11 (8–14)
Naive (CD19+/CD27−)	80 (60–92)	56 (38–83)	46 (41–55)
Memory (CD19+/CD27+)	34 (18–45)	17 (11–25)	24 (10–49)
Plasma (CD19+/CD138+)	3 (1–6)	1.5 (0.5–2.2)	2.7 (0.9–4.9)

NOTE. Data are median (range) percentage of cells that showed staining. Peripheral-blood lymphocytes from filaria-infected individuals with chronic pathology/lymphedema (CP), chronically infected but asymptomatic individuals (CA+), and uninfected individuals (UN) were stained for lineage markers—CD3, CD56, and CD19—as well as for a variety of activation/phenotypic markers and were analyzed by flow cytometry. There were no significant differences between the 3 groups.
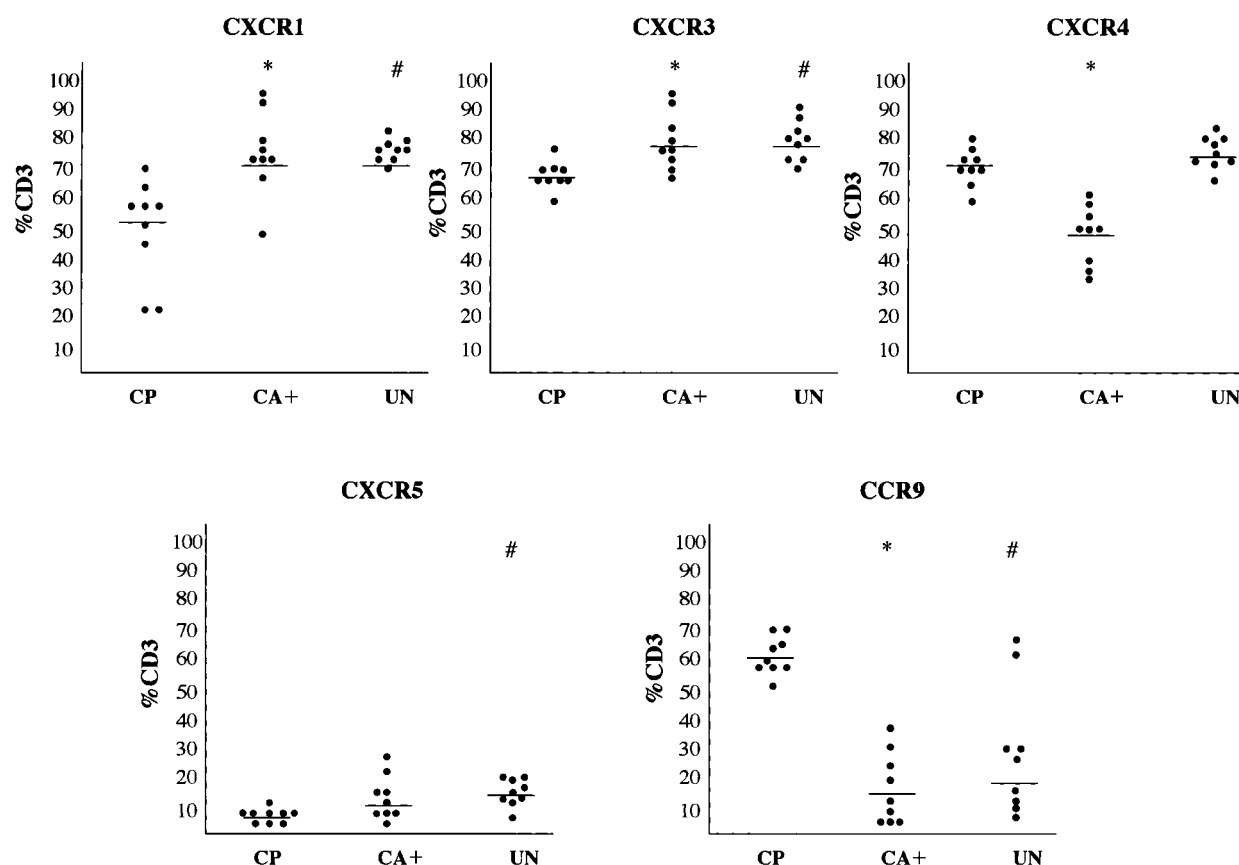
Figure 1. Expression of chemokine receptors on the surface of T cells. Peripheral-blood lymphocytes from filaria-infected individuals with chronic pathology/lymphedema (CP), chronically infected but asymptomatic individuals (CA+), and uninfected individuals (UN) were gated on CD3+/CD56− cells and were stained to detect the expression of chemokine receptors. Results are shown as the percentage of CD3+ cells expressing the given chemokine receptor; the horizontal bars represent the geometric means. *$P < .05$, CP vs. CA+, by Mann-Whitney test. #$P < .05$, CP vs. UN, by Mann-Whitney test.

by use of ACK lysis buffer (Biosource). Cells were then washed and cryopreserved in a medium containing RPMI-1640 (Bio-Whittaker), 10% heat-inactivated fetal calf serum (Harlan Bio-products for Science), and 20% dimethyl sulfoxide (Fisher Scientific). Also, additional serum samples were collected and frozen for further analysis.

Preparation of parasites. Live, infective-stage larvae (L3) and live microfilariae (Mf) of *B. malayi* were provided by Dr. John McCall (University of Georgia, Athens) and were repeatedly washed in RPMI-1640 with antibiotics and were cultured in 5% CO_2 at 37°C.

In vitro culture. PBLs were cultured, with either L3 (5–10/well) or Mf (50,000 /well), in 24-well tissue-culture plates (Corning), at a concentration of 5×10^6 cells/well; 24 h later, cells were washed in a buffer of PBS and 0.1% bovine serum albumin and were stained for flow cytometry.

Reagents for flow cytometry. Antibodies used for phenotype analysis were from BD Pharmingen/BD Biosciences, and antibodies used for analysis of chemokine receptors were from R&D Systems. The following antihuman antibodies were used:

CD69, CD71, CD45RA, CD138, and CD56, labeled with fluorescein isothiocyanate; CD25, CD27, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, and CCR9, labeled with phycoerythrin; were used: CD45RO and CD19, labeled with peridinin chlorophyll protein; and CD3, labeled with allophycocyanin.

Flow cytometry. Cryopreserved PBLs were thawed, were washed in a buffer of PBS and 0.1% bovine serum albumin, and were stained for the presence of various surface markers and chemokine receptors. Fluorescence was measured on a FACSCaliber (Becton Dickinson) by use of 10,000 gated lymphocytes.

ELISA. In serum from 5 individuals in each group, levels of interleukin (IL)–8, RANTES, macrophage inflammatory protein (MIP)–1α, MIP-1β, growth-related oncogene α (GROα), monocyte chemotactic protein (MCP)–1, thymus-and-activation–regulated chemokine (TARC), and interferon-inducible protein (IP)–10 chemokines were measured by use of Search-light Multiplex ELISA Technology (Pierce Biotechnology).

Statistical analysis. The data were compared by use of either the Mann-Whitney test, the Wilcoxon signed-rank test,
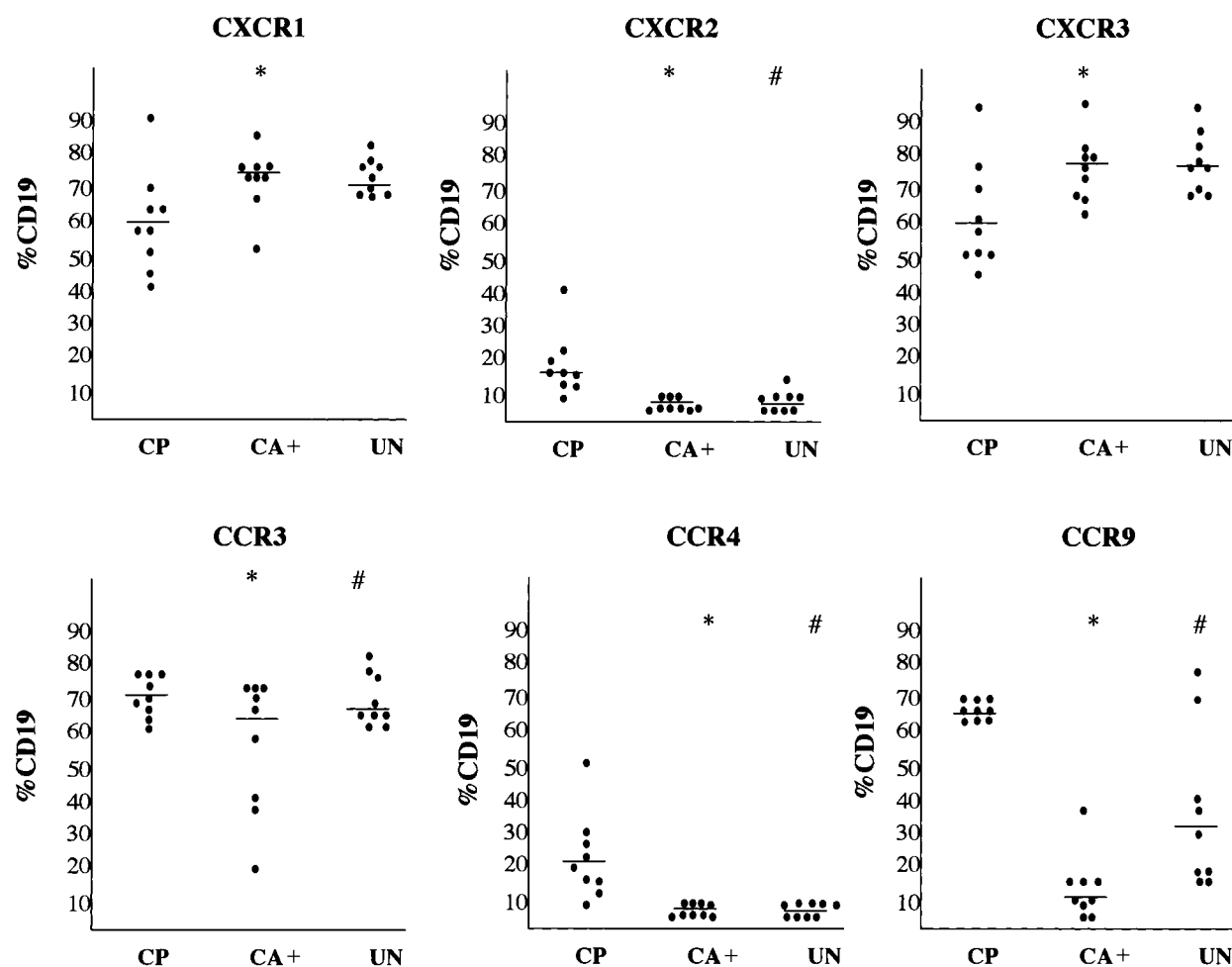
Figure 2. Expression of chemokine receptors on the surface of B cells. Peripheral-blood lymphocytes from filaria-infected individuals with chronic pathology/lymphedema (CP), chronically infected but asymptomatic individuals (CA+), and uninfected individuals (UN) were gated on CD19$^+$ cells and were stained to detect the expression of chemokine receptors. Results are shown as the percentage of CD19$^+$ cells expressing the given chemokine receptor; the horizontal bars represent the geometric means. *$P < .05$, CP vs. CA+, by Mann-Whitney test. #$P < .05$, CP vs. UN, by Mann-Whitney test.

or Spearman rank correlation. All statistics were derived by use of StatView 5 software (SAS Institute).

RESULTS

Absence of intergroup differences in the numbers of T cells and B cells. To assess the impact that the numbers of T cells and B cells had on clinical filarial disease, the relative proportions of T cells (CD3$^+$/CD56$^-$) and B cells (CD19$^+$) in peripheral blood from individuals in the CP, CA+, and UN groups were analyzed; no significant differences were found between the groups (table 2). T cells from individuals in the 3 groups were examined for surface expression of CD69, CD25, and CD71 (all activation markers), CD45RA (a marker of naive T cells), and CD45RO (a marker of effector/memory T cells); no significant differences were detected between the 3 groups (table 2). The percentages of naive B cells (CD19$^+$/CD27$^-$), memory B cells (CD19$^+$/CD27$^+$), and plasma cells (CD19$^+$/CD138$^+$) in

the peripheral blood were determined by use of surface-marker phenotyping; no significant differences were observed between the 3 groups (table 2). Thus, both quantitatively and qualitatively, with respect to markers for activation and for phenotype, T cells and B cells from the 3 groups did not differ significantly.

Expression of chemokine receptors on the surfaces of T cells and B cells. To assess the role that chemokine receptors play in clinical disease, the expression of chemokine receptors on the surface of T cells (gated as CD3$^+$/CD56$^-$) from the 3 groups was examined (figure 1). CP individuals had a significantly higher percentage of CXCR4-expressing T cells ($P = .008$, by Mann-Whitney test) than did CA+ individuals. Even more dramatic was that CP individuals had a significantly higher percentage of CCR9-expressing T cells than did either CA+ individuals ($P = .0003$, by Mann-Whitney test) or UN individuals ($P = .019$, by Mann-Whitney test). Conversely, CP individuals had significantly lower percentages of CXCR1-expressing T cells and of CXCR3-
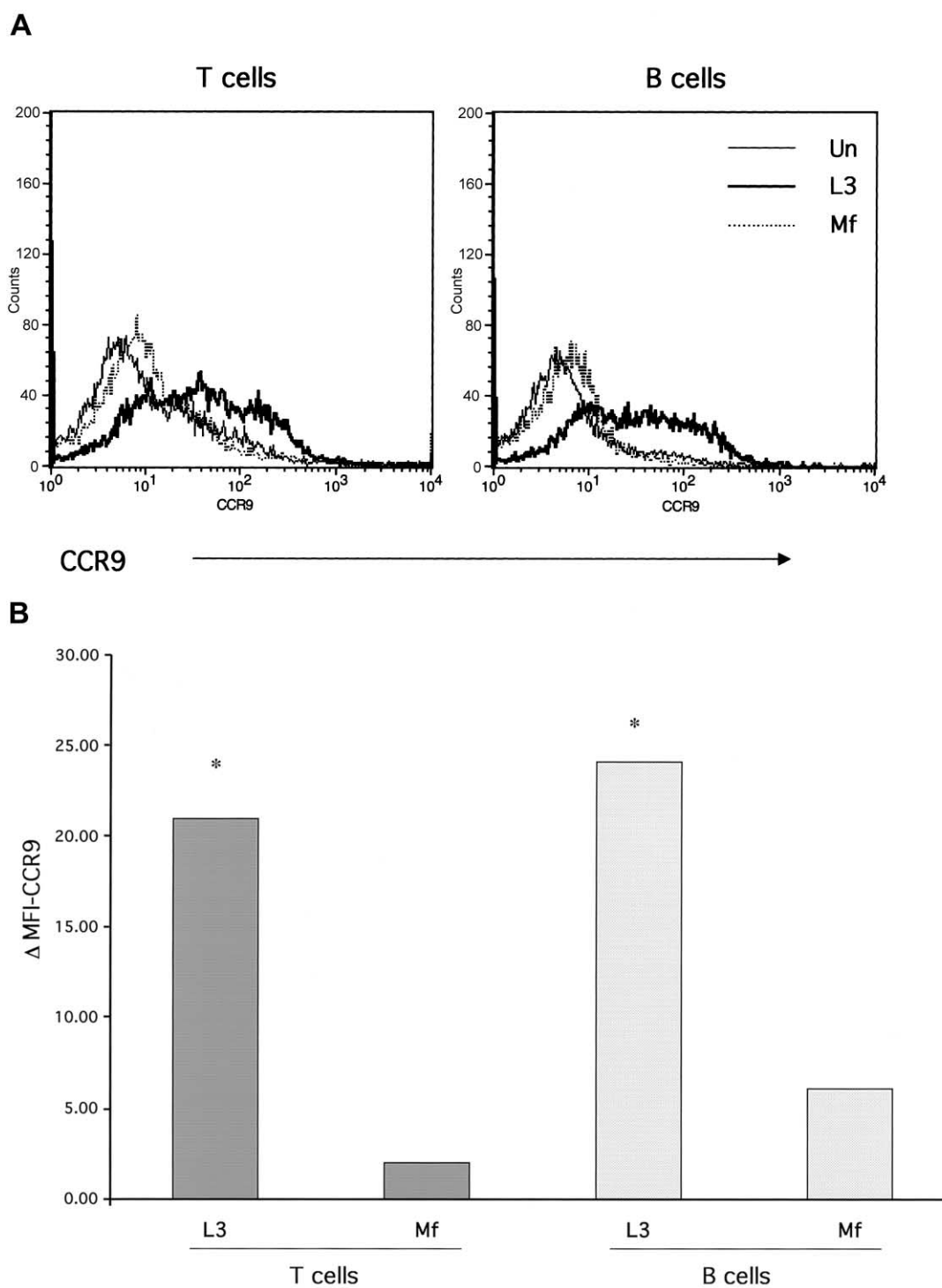
Figure 3. Increased expression of CCR9 in response to live, infective-stage larvae (L3) of *Brugia malayi* in T cells and B cells of filaria-infected individuals with chronic pathology/lymphedema (CP). Peripheral-blood lymphocytes were cultured with either L3, microfilariae (Mf), or media control (Un) and were analyzed 24 h later for the expression of CCR9. *A,* Mean fluorescent intensity (MFI) of the expression of CCR9 on the surface of T cells and of B cells, in 1 representative experiment. *B,* Change, in response to either L3 or Mf, in the MFI of the expression of CCR9 (ΔMFI-CCR9) on the surface of T cells and of B cells in 5 CP individuals, compared with that in control media. MFI-CCR9 was significantly increased in response to L3. *$P = .0431$, response to L3, by Wilcoxon signed-rank test; $P > .05$, response to Mf, by Wilcoxon signed-rank test.
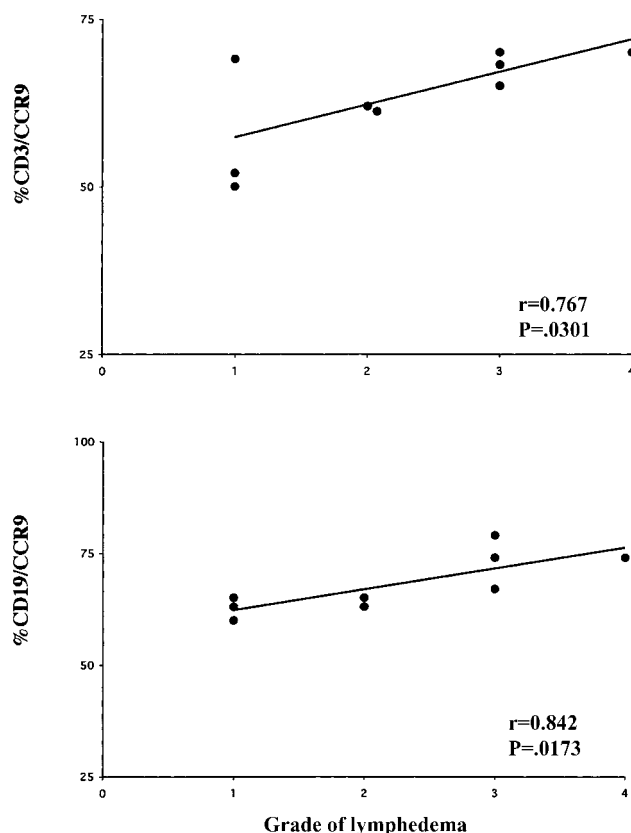
Figure 4. Correlation between expression of CCR9 on peripheral-blood lymphocytes and grade of lymphedema in filaria-infected individuals with chronic pathology/lymphedema (CP). The grade of lymphedema in 9 CP individuals was assessed clinically by use of the standard classification system that has been established by the World Health Organization. The percentages of CCR9-expressing T cells and CCR9-expressing B cells were assessed by use of flow cytometry. Spearman correlation was used to measure the correlation between the variables.

expressing T cells than did either CA+ individuals ($P = .004$ and $P = .0007$, respectively, by Mann-Whitney test) or UN individuals ($P = .001$ and $P = .004$, respectively, by Mann-Whitney test). Finally, CP individuals had a significantly lower percentage of CXCR5-expressing T cells ($P = .009$, by Mann-Whitney test) than did UN individuals. No significant intergroup differences were found in the expression of either CCR3, CCR4, CCR5, CCR6, or CCR7 (data not shown). Furthermore, very little or no expression of CXCR2, CXCR6, CCR1, and CCR2 could be found on the surface of T cells.

The expression of chemokine receptors on the surface of gated B cells (CD19$^+$) was also examined (figure 2). Compared with CA+ individuals and with UN individuals, CP individuals had a significantly higher percentage of B cells that expressed the following chemokine receptors: CXCR2 ($P = .0006$, vs. CA+, by Mann-Whitney test; $P = .002$, vs. UN, by Mann-Whitney test), CCR3 ($P = .011$, vs. CA+, by Mann-Whitney test; $P = .024$, vs. UN, by Mann-Whitney test), CCR4 ($P = .005$, vs.

CA+, by Mann-Whitney test; $P = .002$, vs. UN, by Mann-Whitney test), and CCR9 ($P = .003$, vs. CA+ and UN, by Mann-Whitney test). As in the case of the corresponding categories of T cells, CP individuals also had significantly lower percentages of CXCR1-expressing B cells ($P = .038$, by Mann-Whitney test) and of CXCR3-expressing B cells ($P = .024$, by Mann-Whitney test) than did CA+ individuals.

Regulation of expression of chemokine receptors, in response to L3. To assess the regulation of chemokine receptors by filarial parasites, PBLs from CP individuals ($n = 5$) were stimulated with either L3 or Mf of *B. malayi* for 24 h, and the expression of chemokine receptors CXCR1, CXCR3, and CCR9 was examined. No significant differences were observed in the percentages of either T cells and B cells that expressed CXCR1 and CXCR3 or in the mean fluorescent intensity (density of expression) of the receptors in response to either L3 or Mf, compared with those of unstimulated controls (data not shown); however, both (1) the percentages of CCR9-expressing T cells (data not shown) and CCR9-expressing B cells ($P = .0431$, by Wilcoxon signed-rank test) and (2) the mean fluorescent intensity of CCR9 receptors ($P = .0431$, by Wilcoxon signed-rank test) in response to L3 but not in response to Mf were higher than those of unstimulated controls (figure 3*A* and *B*). We also examined the regulation, in response to L3 and Mf in vitro, of CXCR1, CXCR3, and CCR9 in UN individuals and in CA+ individuals; we found no significant differences from the results seen in unstimulated controls (data not shown).

Expression of CCR9 is possibly associated with the grade of lymphedema seen in CP individuals. To investigate the possible involvement of CCR9 in the development of lymphedema, we examined the correlation between the grade of lymphedema in CP individuals and the percentages of both T cells and B cells that expressed chemokine receptors. The grade of lymphedema, based on the WHO classification system, was significantly correlated with the percentages of both CCR9-expressing T cells ($P = .03$, by Spearman correlation) and CCR9-expressing B cells ($P = .017$, by Spearman correlation) (figure 4).

Levels of chemokines in serum are significantly higher in CP individuals than in UN individuals. To assess the role that chemokines (CCR and CXCR ligands) play in the pathogenesis of filarial disease, the levels of selected chemokines in serum were measured in a subset ($n = 5$) of individuals in each group. The levels of IL-8 ($P = .009$, by Mann-Whitney test), MIP-1α ($P = .047$, by Mann-Whitney test), MIP-1β ($P = .009$, by Mann-Whitney test), MCP-1 ($P = .009$, by Mann-Whitney test), TARC ($P = .028$, by Mann-Whitney test), and IP-10 ($P = .009$, by Mann-Whitney test) were significantly higher in CP individuals than in UN individuals (figure 5). No intergroup differences in the levels of either RANTES or GROα were observed. It is of interest is that, for all the chemokines examined,
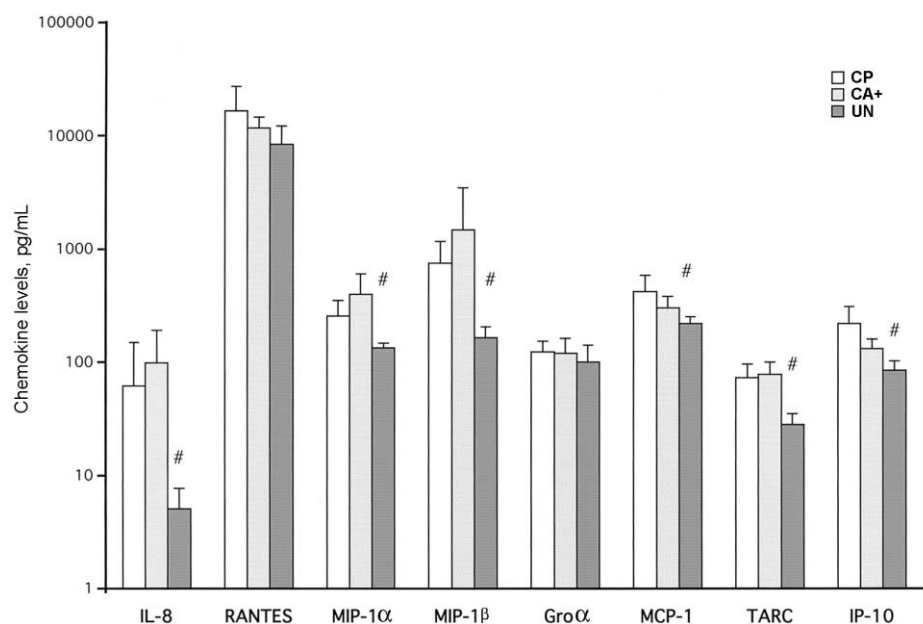
Figure 5. Levels of chemokines in serum from 3 clinical groups. Serum from filaria-infected individuals with chronic pathology/lymphedema (CP), chronically infected but asymptomatic individuals (CA+), and uninfected individuals (UN) was analyzed, by multiplex ELISA, for levels of chemokines. Geometric means and 95% confidence intervals of levels of chemokines are shown for 5 CP *(white bars),* 5 CA+ *(dotted bars),* and 5 UN *(shaded bars)* individuals. #$P < .05$, by Mann-Whitney test; CP vs. UN. IL-8, interleukin 8; MIP-1α, macrophage inflammatory protein 1α; MIP-1β, macrophage inflammatory protein 1β; GROα, growth-related oncogene α; MCP-1, monocyte chemotactic protein 1; TARC, thymus-and-activation–regulated chemokine; IP-10, interferon-inducible protein 10.

the levels in serum were not significantly different in CP individuals compared with CA+ individuals.

DISCUSSION

In areas in which lymphatic filariasis—an infectious disease with a spectrum of clinical manifestations—is endemic, individuals most commonly fall into the 3 categories: infected subjects free of overt clinical disease (whom the present article has denoted "CA+"); patients with chronic disease manifestations with or without active infection (whom the present article has denoted as "CP"); and individuals free of both infection and disease (whom the present article has denoted as "UN") [1]. The most debilitating consequences of filarial infection are lymphedema and elephantiasis, which have significant economic and psychosocial effects on both the infected individual and the community [14]. Indeed, the global burden of lymphatic filariasis has been estimated as a loss of 5.549 million disability-adjusted life-years (a loss exceeded only by that for malaria and that for tuberculosis) [14]. The lymphatic damage in filariasis is thought to evolve as a consequence of either mechanical damage or dysfunction associated with live parasites, bacteria-induced chronic inflammation, and host immune responses [3]. To gain insight into the pathogenesis of lymphedema, we examined the expression of chemokine receptors by cells likely to infiltrate the site of pathology.

We first estimated quantitative and qualitative differences in T cells and B cells from CP individuals, from CA+ individuals, and from UN individuals. We found no intergroup differences in the relative proportions of T cells and B cells or the expression of either activation or phenotypic markers. Our analysis included study of T cell–activation markers (CD69, CD25, and CD71) and T cell–naive and effector/memory markers (CD45RA/CD45RO). We also found no intergroup differences in the proportions of naive B cells, memory B cells, and plasma cells in the peripheral blood. These results suggest that the intergroup differences in clinical manifestations cannot be attributed to obvious differences in either the numbers/percentages or the activation/differentiation states of T cells and of B cells.

We then examined the expression of CXC and CC chemokine receptors on the surface of T cells and of B cells. We found significantly lower percentages of both T cells and B cells that expressed CXCR1 and CXCR3 and significantly higher percentages of both T cells and B cells that expressed CCR9 in CP individuals, compared with those in CA+ and UN individuals. CXCR1 and CXCR3 are important receptors for recruitment of Th1 cells, and CCR9 is a marker of gut-homing lymphocytes [15, 16]. The only known ligand for CCR9, thymus-expressed chemokine (TECK), is a tissue-specific chemokine expressed predominantly in the epithelium of the small intestine and on endothelial surfaces [17]. We hypothesize that expression of TECK on lymphatic endothelium directs CCR9-expressing T cells and CCR9-expressing B cells to the site of lymphatic pa-

thology. In further confirmation of our hypothesis, we found, in the 9 CP individuals, a significant correlation between the grade of lymphedema and the percentages of CCR9-expressing T cells and CCR9-expressing B cells.

We next examined the regulation, in response to L3 and Mf, of CXCR1, CXCR3, and CCR9. We observed that, in T cells and B cells of CP individuals, CCR9 is significantly up-regulated in response to L3 but is not up-regulated in response to Mf. This observation suggests that the trigger for the development of lymphatic damage in individuals predisposed to lymphatic pathology might be the increase, in response to incoming larvae, of CCR9-expressing T cells and CCR9-expressing B cells. Analyses of the regulation of CCR9 in CA+ individuals and in UN individuals revealed no changes in response to either L3 or Mf, indicating that up-regulation of CCR9 is a unique property of CP individuals. Neutralizing antibodies or small-molecule antagonists are being used to inhibit chemokine receptors in experimental treatments of a variety of disorders—for example, CCR3 for asthma [18], CCR1 for experimental allergic encephalitis [19], CXCR3 for cardiac allograft rejection [20], and CCR5 for HIV infection [21]. Thus, it is conceivable that CCR9, used as an antagonist, would present a potent weapon for the prevention of lymphedema in areas where filariasis is endemic.

An unrelated but interesting observation in our studies was the lower percentage of CXCR4-expressing T cells in CA+ individuals, compared with that in CP individuals and that in UN individuals. Because CXCR4 is a coreceptor for T-trophic HIV [22], this expression pattern might render CP individuals and CA+ individuals less susceptible to HIV infection than UN individuals are, although data from a different cohort of filaria-infected patients suggest otherwise [23]. Finally, we examined the levels of different chemokines in serum from a subset of our 3 groups. The levels of IL-8, MIP-1α, MIP-1β, MCP-1, TARC, and IP-10 were significantly higher in CP individuals than in UN individuals It is noteworthy that IL-8 is a ligand for CXCR1, IP-10 is a ligand for CXCR3, and TARC is a ligand for CCR4, and all of these chemokine receptors are expressed at significantly different levels in CP individuals, compared with those in UN individuals. Hence, a chemokine/chemokine-receptor network that involves these receptor-ligand pairs might be operational in the disease process of filariasis.

In conclusion, our results suggest that, in CP individuals, there is an augmented expression of CCR9 and a diminished expression of both CXCR1 and CXCR3, compared with those in CA+ individuals and those in UN individuals. Our results also reveal a significant correlation between the expression of CCR9 and the grade of lymphedema in CP individuals. Finally, the expression of CCR9 in response to L3 is significantly higher in CP individuals, compared with that in unstimulated controls. Together, these data highlight the possible participation of the chemokine network in the pathogenesis of lymphedema and could provide us with tools to manipulate the immune system to prevent debilitating pathology.

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